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Isolation and Purification of Ceramide-aminoethylphosphonate in the AWABI, *Haliotis discus*

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ABSTRACT

Ceramide-aminoethylphosphonate was isolated from the AWABI, *Haliotis discus*.

The compound was purified with a combination of mild alkaline hydrolysis of the total lipids, QAE-Sephadex -A25 column chromatography and two dimensional thin layer chromatography.

The infrared spectrum showed an absorption band at 1180 cm^{-1} due to c-p bond, and was essentially identical with that of the standard ceramide aminoethylphosphonate. Upon hydrolysis of the substance by strong acid, neither change in the chromatographic behaviors of this substance nor liberation of inorganic phosphate was observed. The stability of the compound to acid hydrolysis suggested the presence of a c-p bond.

On comparison with synthetic compound, the aqueous hydrolysis product behaved like 2-aminoethylphosphonic acid on thin layer chromatograms. The predominant fatty acids were palmitic acid, oleic acid, arachidonic acid, eicosapentaenoic acid and docosahexaenoic acid.

INTRODUCTION

Natural compounds containing a carbon-phosphorus bond were first detected when Horiguchi and Kandatsu[1] isolated 2-aminoethylphosphonic acid from ciliate protozoa in the rumen of sheep. Since then this compound has been shown to be present either in the free form or in the bound to proteins or lipids in the protozoan *Tetrahymena pyriformis*, fresh water molluscus, terrestrial mammals, and marine animals in the phyla coelenterate, mollusca, echinoderamata. So far, phosphonate-containing lipids (termed phosphonolipid[2]) have been found as analogs of phosphatidylethanolamine, phosphatidylcholine and sphingoethanolamine from tissue of lower[3] and higher animals[4]. Rouser et al[5] first isolated a characteristic lipid from

sea anemone by means of column chromatography, and it was assumed tentatively to consist of a ceramide attached to 2-aminoethylphosphonic acid by an ester linkage. Since then this ceramide aminoethylphosphonate has been shown to be widely present in shellfish both in fresh water and sea water[6], and Fungi[7].

In our previous paper, we reported that ceramide-aminoethylphosphonate was isolated from the AGEMAKI, *Sinonovacula constricta*[8], JANBOTANISHI, *Ampullarius urceus*[9] and GAZAMI, *Portunus trituberculatus*[10]. So far, there is still no evidence of either isolation or purification of ceramide-aminoethylphosphonate from AWABI, *Haliotis discus*.

Therefore, the present investigation was undertaken to isolate of the ceramide-aminoethylphosphonate from the AWABI, *Haliotis discus*.

MATERIALS AND METHODS

Materials:

QAE-Sephadex-A25 was the product of Pharmacia. 2-aminoethylphosphonic acid was prepared by the method described by Kosolapoff[11].

Fractionation of Phosphorus-fraction of the AWABI, Haliotis discus:

Fractionation of phosphorus fraction (A: acetone soluble, B: chloroform-methanol soluble, C: TCA soluble, D: TCA insoluble) from the freeze dried material of the AWABI, Haliotis discus was carried out by the method as previously described[8]. A portion of each fraction was hydrolyzed with 6N HCl at 120°C for 24 hrs. The hydrolysate was treated several times with ether. The aqueous layer was filtered, evaporated to dryness, and redissolved with water for analysis of phosphonate- and total-phosphorus.

Isolation of Ceramide Aminoethylphosphonate:

Lipid of the freeze dried material(20g) of the AWABI, Haliotis discus was extracted by the method as previously described[8]. The combined extracts was washed according to the method described by Folch et al[12]. The chloroform layer was evaporated to dryness in a N_2 stream, and the isolation and purification of the sphingophosphonolipid from the lipid was achived by three methods as previously described[8]: by the mild alkaline hydrolysis according to Dowson[13] and Hori[14], by the QAE-Sephadex column chromatography and by the preparative thin layer chromatography.

Infrared Analysis:

The infrared absorption spectrum was determined on a pellet of potassium bromide using a Nihonbunko IR-S Spectrophotometer.

Qualitative Analysis:

The ceramide aminoethylphosphonate was hydrolyzed with 6N HCl at 120°C for 24 hrs. The liberated fatty acids were extracted with light petroleum and the water soluble compounds were extracted by Folch's partition.

The aqueous layer was evaporated to dryness under reduced pressure.

The presence of aminoethylphosphonate in the hydrolysate was demonstrated by thin layer chromatography (Kiesel gel $60F_{254}$, 20×20cm, 0.25mm) using five different solvent systems[15].

Authentic 2-aminoethylphosphonic acid was co-chromatographed and the spots were detected by both ninhydrine and Rosenberg's method[16].

Quantitative Analysis:

Fatty acid ester were obtained from the ceramide aminoethylphosphonate by methanolysis (5% HCl in methanol at 100°C for 4 hrs) and subjected to gas chromatography. Phosphonate phosphorus was estimated by the method of Tamari et al[17]. Total phosphorus was estimated by the method of Chen et al[18].

Gas Chromatography of Fatty Acid:

Fatty acid methyl esters were separated on a Shimadzu GC-8A apparatus equipped with a flame ionization detector. The column was a 3mm×2m stainless column packed with 5% Shinchron E7I on 80-100 mesh Chimalite, and the instrument was operated at 200°C. The peaks were identified by comparison with those of standard methyl esters on the basis of individual retention time.

RESULTS

In Table 1 is reported the phosphonate content of the phosphorus fractions of the AWABI, *Haliotis discus*. The phosphonate content in the A, B, C and D fractions were 107.1, 12.5, 0.04 and 112.4 mg per 100g of the AWABI, Haliotis discus, respectively. The A, B, C and D fractions contained 46.2%, 5.4%, 0.02% and 48.4% of the total phosphonate in the AWABI, *Haliotis discus*.

Table 1. Phosphonate Contents in the Phosphorus Fractions of the AWABI, *Haliotis discus*.

Fraction	C-P (mg/100g)	T-P (mg/100g)	C-P/T-P (%)
A	107.1 (46.2)	371.9	28.8
B	12.5 (5.4)	85.4	14.6
C	0.04(0.02)	30.3	0.1
D	112.4 (48.4)	465.6	24.1

C-P: Phosphonate-phosphorus, T-P: Total-phosphorus,
( ): as % of total C-P

About 28.8%, 14.6%, 0.1% and 24.1% of the total phosphorus was found as phosphonate in the A, B, C and D fractions. A typical thin layer chromatographic composition of phospholipids classes was identical to that of other invertebrates[19].

Figure 1 shows the thin layer chromatogram of the sphingolipid obtained by the mild alkaline hydrolysis method. Ceramide aminoethylphosphonate (spot 1), and probably what is hydroxy fatty acid containing ceramide aminoethylphosphonate (spot 2) and sphingomyelin (spot 3), consisted of 40.2, 9.2 and 43.1% of the sphingolipid phosphorus, respectively. The result obtained here indicates that ceramide aminoethylphosphonate is the major sphingolipid component of the lipid moiety of the AWABI, Haliotis discus.

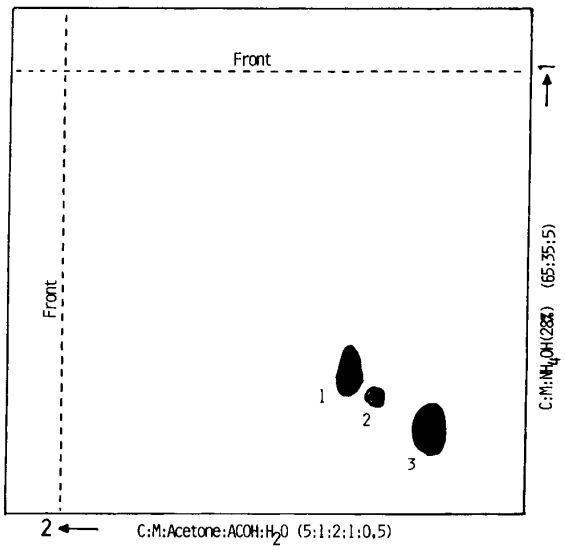

Fig. 1 Two Dimensional Thin Layer Chromatogram of the Alkaline Stable Lipids Obtained by the Mild Alkaline Hydrolysis as Previously Described[8]. The chromatogram was developed as previously described[8].
Spot are: 1, ceramide aminoethylphosphonate (40.2%); 2, probably hydroxy fatty acid containing ceramide aminoethylphosphonate (9.2%);3, sphingomyelin (43.1%)

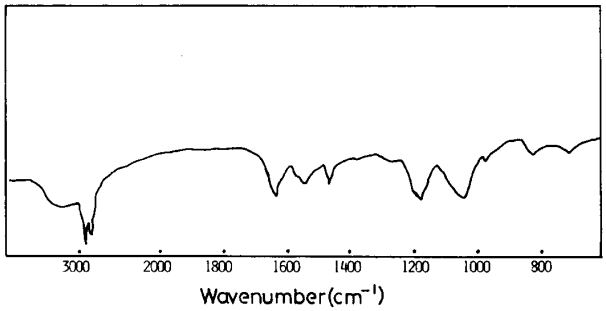

Fig. 2 Infrared Spectrum of the Ceramide Aminoethylphosphonate Purified by a Combination of the Mild Alkaline Hydrolysis, the Column Chromatography and the Preparative Thin Layer Chromatography as Previously Described[8].

Further, ceramide aminoethylphosphonate was purified by QAE-sephadex column chromatography from the sphingolipids obtained by mild alkaline hydrolysis of the phospholipid fraction, and pure ceramide aminoethylphosphonate was then obtained by preparative thin layer chromatography.

Figure 2 shows the infrared spectrum of the ceramide aminoethylphosphonate obtained by the alkaline hydrolysis method. The infrared spectrum lacked a band at 1730-1750cm^{-1}, indicating the absence of ester bonds. A band was present at 1180cm^{-1}, indicating the presence of carbon-phosphorus bond, and was essentially identical with that of ceramide aminoeth-

Table 2. Rf Values on Thin Layer Chromatography of Authentic 2-Aminoethylphosphonic acid and Compound Isolated from AWABI, *Haliotis discus*.

	Solvent				
	1	2	3	4	5
Authentic Compound	0.59	0.36	0.13	0.13	0.13
Isolated Compound	0.58	0.36	0.12	0.14	0.13

1. Ethanol: 7% ammonia (1: 2, v/v)
2. Isopropanol: acetic acid: 15% ammonia: water (5: 2: 4: 3, v/v)
3. Methanol: formic acid: water (16: 3: 1, v/v)
4. 0.02N acetic acid in 60% ethanol
5. Methanol: pyridine: water (20: 1: 5, v/v)

Table 3. Fatty acid Composition of the Ceramide Aminoethylphosphonate Isolated from the AWABI, *Haliotis discus*.

Fatty acid	Ceramide-aminoethylphosphonate (%)
14 : 0	4.2
16 : 0	23.1
18 : 0	9.2
18 : 1	18.1
18 : 2	2.6
18 : 3	2.7
20 : 0	2.2
20 : 3	2.3
20 : 4	11.2
20 : 5	15.2
22 : 6	9.2

ylphosphonate, obtained by Rouser et al[5], Hayashi et al[20], and Hori et al[14,21].

The carbon-phosphorus compound in the acid hydrolysate of the ceramide aminoethylphosphonate was found to be 2-aminoethylphosphonic acid by thin layer chromatography with five different solvent systems (Table 2). Inorganic phosphorus and N-methyl-derivatives of 2-aminoethylphosphonic acid were not detected in the hydrolysate. The stability of the compound to acid hydrolysis suggested the presence of a C-P bond.

The results of gas chromatographic analysis of fatty acid composition are shown in the Table 3. The fatty acid composition was very simple.

The fatty acids of the ceramide aminoethylphosphonate were characterized by relatively high percentages for palmitic acid, oleic acid, arachidonic acid, eicosapentaenoic acid and docosahexaenoic acid.

The sum of these four acids comprised about 77% of total fatty acids.

From the above results, the isolated phosphonolipid was identified as the ceramide

2-aminoethylphosphonate.

DISCUSSION

The distribution of phsphonolipids in nature is primarily limited to lower animals such as mollusca, coelenterates and protozoa, although the lipids have also been found in small quantities in mammalian tissues[22-29].

In the phosphonolipids, it has been established that glycero-aminoethylphosphonate (GPnL) occurs as the major phospholipid of the ciliary membranes of protozoan species[25,26]. To date, there is still no report on the detection of the GPnL in shellfish and sea anemones.

On the other hand, ceramide aminoethylphosphonate are found in high concentration in mollusca, coelenterates and shellfish, while the concentration is low in *Tetrahymena*. Matsubara[27] found that the oyster adductor muscle contained the highest concentration (45% of the total sphingolipids) of ceramide aminoethylphosphonate.

Komai et al[28] found that ceramide-aminoethylphosphonate occurs in approxymately 11% of the phospholipids in the nervous system of *A. Kurodai* a marine gastropod.

Moreover, Komai et el speculate that ceramide aminoethylphosphonate may be indispensible in shellfish for neuronal function. Kittredge et al[29] indicate that the presence of a covalent carbon-phosphorus linkage in the anemone has been postulated as functionally analogous to a fixation process.

Mason[30] indicates the possibility that the presence of highly ionic lipid such as ceramide aminoethylphosphonate could play a direct role in facilitating the transport of small ions from the aqueous emvironment into the intercellular space of the anemone.

On the other hand, the occurrence of the ceramide aminoethylphosphonate in the sea-urchin gonad[31] and fresh water mussel spermatozoa[19] and egg[32,33] has been reported. While this sphingophosphonolipid is unique to the invertebrates, its exact function is unknown, but their resistance to endogenous hydrolytic enzymes is highly suggestive of a protective function.

It will be of further interest to determine of the presence of ceramide aminoethylphosphonate is localized in the shellfishs.

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